A SIMPLE METHOD FOR THE SILVER IMPREGNATION OF RETICULUM *

HAROLD GORDON, M.D., M.S., AND HENRY H. SWEETS, JR., B.S.

(From the Department of Pathology, University of Louisville, School of Medicine, Louisville, Ky.)

Silver staining methods, long adopted as a standard procedure by neuropathologists, are not yet in common use for purposes of routine tissue diagnosis. Four general objections have been raised to the use of silver as a staining agent: (1) the uncertain results, (2) the need of special fixatives, (3) the use of frozen section technic, and (4) unfamiliarity with the pictures obtained. Kubie and Davidson [1] and Foot [2] have provided exact formulas to replace the empirical solutions devised by Bielschowsky,[3] Ramón y Cajal,[4] and Río-Hortega.[5] The equimolar solutions advocated by Kubie and Davidson [1] provide the chemical basis needed to ensure uniform results. With a little experience and attention to detail, silver stains can be made to yield results as constant as do the acid and basic dyes in common use. Foot,[6] and Foot and Foot [7] are largely, though not solely, responsible for making formalin-fixed and paraffin embedded tissues available for silver impregnation. The fourth objection is the least valid. The very specificity of the argyrophil reaction is one of the strongest arguments for its adoption in the field of "general" pathology. The silver methods provide an excellent supplement to the dye stains. To Masson [8, 9, 10] belongs much of the credit for stimulating interest in the wider application of the silver impregnation methods and showing their value in research and diagnosis.

Silver impregnation lends itself especially well to the demonstration of the reticular connective tissues. Perdrau,[11] Foot and Mènard,[12] and Laidlaw [13, 14] have devised useful methods for impregnating connective tissue with silver. Perdrau's formula yields almost uniformly constant results. While his method can be recommended as a standard of comparison for the evaluation of new methods, it is too tedious and time-consuming to lend itself to routine application. The method devised by Foot and Mènard [12] is rapid and applicable to tissues fixed either in Zenker's solution or in formalin. However, the results are not uniformly satisfactory. A series of variants re-

* Received for publication December 20, 1935.

cently introduced by Foot and Foot [7] call for confusing variations in technic and, where it is desired to demonstrate particularly the connective tissues, offer no advantages over the older methods. Laidlaw [13] used a chemically unbalanced and highly concentrated solution of silver diammino carbonate. This is not an easy solution to work with. Therefore, there seems place for the following method which has been found to yield results as certain and constant as the Perdrau technic.

Method

Fixation: Either 10 per cent aqueous formalin solution (4 per cent formaldehyde) or Bouin's fixative may be used. The formalin may be neutral or not. Tissues should be well fixed. They may be embedded in paraffin or in celloidin or cut on the freezing microtome. Celloidin sections may be used as such. Frozen sections should be affixed to slides or coverslips by means of a thin coating of celloidin (Wright's technic). Paraffin sections are transferred to slides or coverslips previously cleaned with strong nitric acid and with alcohol. Mayer's egg albumin medium does not effectively prevent detachment of the sections in the strongly alkaline silver impregnation solution. Three alternative methods are available, instead, for the protection of the sections during impregnation:

(1) The paraffin sections are floated on clean slides or coverslips and dried in the incubator. The slides are transferred, in turn, to xylol, absolute alcohol, and to a mixture of absolute alcohol and ether (equal parts). They are then immersed in thin celloidin, drained and partially dried in air. The celloidin film is hardened in 70 per cent alcohol and the sections placed in water (Wright's technic).

(2) The paraffin sections are floated on to a warm 1 per cent aqueous solution of gelatin, transferred to slides or coverslips and allowed to drain. The gelatin is then hardened by exposure to formaldehyde vapor. This is a modification of the Masson gelatin glue method [15] and is especially recommended for sections of the central nervous system. It is also useful for frozen sections where it is desired to demonstrate lipoids, using Scharlach R or Sudan III as a counterstain.

(3) Warthin's molasses and celloidin sheet principle [16] may be

used. This is especially advantageous when many sections are cut from a single block of tissue.

Oxidation and Reduction: A modification of the well known Mallory bleach is employed. The sections are oxidized for 1–5 minutes in an acidified potassium permanganate solution as follows: to 47.5 cc. of 0.5 per cent aqueous potassium permanganate solution add 2.5 cc. of 3 per cent sulphuric acid. After a brief wash in water, bleach until white in 1 per cent oxalic acid, then wash, in rapid succession, in tap water and in 2 or 3 changes of distilled water.

Mordanting: The sections are placed in 2.5 per cent aqueous solution of ferric ammonium sulphate (iron alum), employed as a sensitizing agent. They may be left in for a variable period (15 minutes to as long as 2 hours). The alum solution may be used repeatedly. After mordanting, the sections must be washed thoroughly in 2 or 3 changes of distilled water.

Impregnation: The diammino silver hydroxide solution of Kubie and Davidson [1] is used. To 5 cc. of 10.2 per cent aqueous silver nitrate solution add strong ammonium hydroxide solution, drop by drop, until the precipitate is just dissolved. Add 5 cc. of 3.1 per cent sodium hydroxide to the ammoniated silver solution, redissolve the resultant precipitate with a drop or two of strong ammonium solution and dilute to 50 cc. with distilled water.

The length of time of impregnation may be gauged by the time it takes the sections to become transparent. This usually occurs almost instantly. Longer impregnation causes the sections to turn a rich brown color and produces an intensely black stain after reduction of the silver. If impregnation is allowed to take place for the optimum period, only a brief wash in distilled water is necessary previous to reduction. If the time of impregnation is prolonged, the sections should be washed more deliberately.

Reduction: An aqueous 10 per cent formalin (4 per cent formaldehyde) is used for this purpose. The sections should be moved to and fro in this solution and reduction is complete almost immediately. After reduction the sections may be washed in tap water.

Toning: Gold toning is merely a refinement and may be omitted. The sections are toned in 0.2 per cent aqueous gold chloride solution (Merck's yellow gold chloride) until they turn a purplish color. The gold solution may be used repeatedly if protected from the "hypo"

fixing solution. After a brief wash the sections are fixed in 5 per cent aqueous sodium thiosulphate ("hypo") and then washed in 2 or more changes of tap water.

Dehydration and Clearing: Sections attached to slides with celloidin are dehydrated in 95 per cent and absolute alcohol. The celloidin is then dissolved in a solution of absolute alcohol and ether, in equal parts. This latter step is not absolutely necessary and is attended by the risk of detachment of the sections from the slides, but it is recommended because surface precipitates are thereby removed, leaving a more sharply impregnated section. The sections are then cleared as usual in xylol and mounted in balsam. Sections fastened to slides with Masson's gelatin glue are dehydrated in 95 per cent and in absolute alcohol, cleared in xylol and mounted in balsam. Celloidin sections, whether embedded as such or secondarily converted to that form by Warthin's celloidin sheet method, are partially dehydrated in 95 per cent alcohol, transferred to carbol-xylol (xylol 2 parts, melted phenol crystals 1 part), cleared in xylol and mounted in balsam.

Results

The reticulum is sharply impregnated, appearing brownish black in untoned, and dark purple in toned preparations. Nerve fibers are also impregnated, appearing almost black, whether toned in gold or not. Elastic fibers are jet black in the toned and in the untoned sections. Cartilage and the trabeculae of decalcified bone assume a pleasing golden brown color in the untoned, a purple gray in the toned preparations. If the processes of oxidation and reduction are properly completed, the background is almost colorless, the cell nuclei and cytoplasm (other than fibers) being silver-negative.

Comment

This method permits impregnation of the fibers in approximately 20 to 30 minutes. In speed alone it does not quite compare with the method recently published by Wilder.[17] However, it permits impregnation of the finest fibers and does not call for many precise or rapid changes of the sections from one solution to another. One of its special features is its lability. The sections may be left in the alum for several hours without spoiling; they may be washed for an

indefinite period at any stage previous to impregnation in the diammino silver hydroxide solution; and they need watching and timing only while being reduced and while being impregnated in the diammino silver solution. The action of the alum mordant is peculiar also in this respect — sections that have been overstained because of prolonged impregnation may be completely destained, after reduction in the formol, by replacing them in the alum solution. Then, after a brief wash, they may be reimpregnated in the diammino silver solution and reduced in formalin. The sections will then be found as well stained as ever. This use of the alum solution is almost a duplicate of its utilization in the Spielmeyer myelin sheath stain.[18] However, the alum will not destain sections once they are toned in gold and fixed in "hypo." The acidified permanganate solution ensures rapid and complete oxidation of the tissues and is an improvement upon the 0.25 per cent aqueous permanganate solution commonly employed for this purpose. Oxalic acid is used for reduction because it is relatively cheap and is quite effective.

Summary

A method, utilizing iron alum as a mordant and, if necessary, as a destaining agent, is suggested for rapid and effective silver impregnation of reticulum of tissues fixed in formalin or in Bouin's solution. The method may be conventionally summarized as follows:

1. Fix in 10 per cent aqueous formalin or in Bouin's solution.
2. Cut frozen sections or embed blocks in paraffin or in celloidin. Affix frozen or paraffin sections to slides by Wright's technic or by Masson's gelatin glue method, or ensheath in celloidin by Warthin's molasses-celloidin sheet method.
3. Oxidize for 1–5 minutes in acidified permanganate solution: 47.5 cc. of 0.5 per cent aqueous potassium permanganate plus 2.5 cc. of 3 per cent sulphuric acid.
4. Wash in water.
5. Bleach until white in 1 per cent oxalic acid.
6. Wash in tap water and 2 changes of distilled water.
7. Mordant for 15 to 30 minutes (or longer) in 2.5 per cent aqueous iron alum.
8. Wash in 2 or 3 changes of distilled water.
9. Impregnate for a few seconds in diammino silver hydroxide.

10. Wash briefly in distilled water.
11. Reduce in 10 per cent aqueous formalin.
12. Wash in water. (If the sections are overimpregnated repeat the process from step 7.)
13. Tone in 0.2 per cent yellow gold chloride 1–3 minutes.
14. Wash in tap water.
15. Fix in 5 per cent sodium thiosulphate 5 minutes.
16. Wash well in tap water.
17. Dehydrate in 80 per cent and in 95 per cent alcohol.
18. (*a*) For sections affixed by Wright's method. Complete dehydration in absolute alcohol and dissolve celloidin in equal parts of absolute alcohol and ether.

18. (*b*) For celloidin or celloidin sheet sections. Complete dehydration in carbol-xylol (xylol 2 parts, phenol 1 part).

19. Clear in xylol.
20. Mount in balsam.

REFERENCES

1. Kubie, Lawrence S., and Davidson, David. The ammoniacal silver solutions used in neuropathology; their staining properties, chemistry and methods of preparations. *Arch. Neurol. & Psychiat.*, 1928, **19**, 888–903.
2. Foot, Nathan Chandler. Comments on the impregnation of neuroglia with ammoniacal silver salts. *Am. J. Path.*, 1929, **5**, 223–238.
3. Bielschowsky, M. Die Silberimprägnation der Axencylinder. *Neurol. Centralbl.*, 1902, **21**, 579–584.
4. Ramón y Cajal, Santiago. Manual de histologia normal y de técnica micrográfica para uso de estudiantes. N. Moya, Madrid, 1910, Ed. 5.
5. Río-Hortega, P. del. Noticia de un nuevo y fácil metodo para la coloración de la neuroglia y del tejido conjuntivo. *Trab. del lab. de inv. biol. de la Univ. de Madrid*, 1917, **15**, 367–378.
6. Foot, Nathan Chandler. Suggestions for staining tumors of spongioblastic origin. *Am. J. Path.*, 1929, **5**, 215–222.
7. Foot, Nathan Chandler, and Foot, Ellen Bellows. A technique of silver impregnation for general laboratory purposes. *Am. J. Path.*, 1932, **8**, 245–254.
8. Masson, P. Contribution to the study of the sympathetic nerves of the appendix. The musculonervous complex of the submucosa. *Am. J. Path.*, 1930, **6**, 217–233.
9. Masson, P. The significance of the muscular "stroma" of argentaffin tumors (carcinoids). *Am. J. Path.*, 1930, **6**, 499–514.
10. Masson, P. Experimental and spontaneous schwannomas (peripheral gliomas). *Am. J. Path.*, 1932, **8**, 367–388, 389–416.

11. Perdrau, J. R. The silver reduction method for the demonstration of connective-tissue fibres. *J. Path. & Bact.*, 1921, **24**, 117.

12. Foot, N. C., and Mènard, M. C. A rapid method for the silver impregnation of reticulum. *Arch. Path. & Lab. Med.*, 1927, **4**, 211–214.

13. Laidlaw, George F. Silver staining of the skin and of its tumors. *Am. J. Path.*, 1929, **5**, 239–247.

14. Laidlaw, George F. Silver staining of the endoneurial fibers of the cerebrospinal nerves. *Am. J. Path.*, 1930, **6**, 435–443.

15. Masson, P. Diagnostics de Laboratoire, Paris, 1923. (Quoted by Laidlaw, G. F., Ref. 13.)

16. Warthin, Alfred Scott. Practical Pathology for Students and Physicians. George Wahr, Ann Arbor, 1921, 245.

17. Wilder, Helenor Campbell. An improved technique for silver impregnation of reticulum fibers. *Am. J. Path.*, 1935, **11**, 817–819.

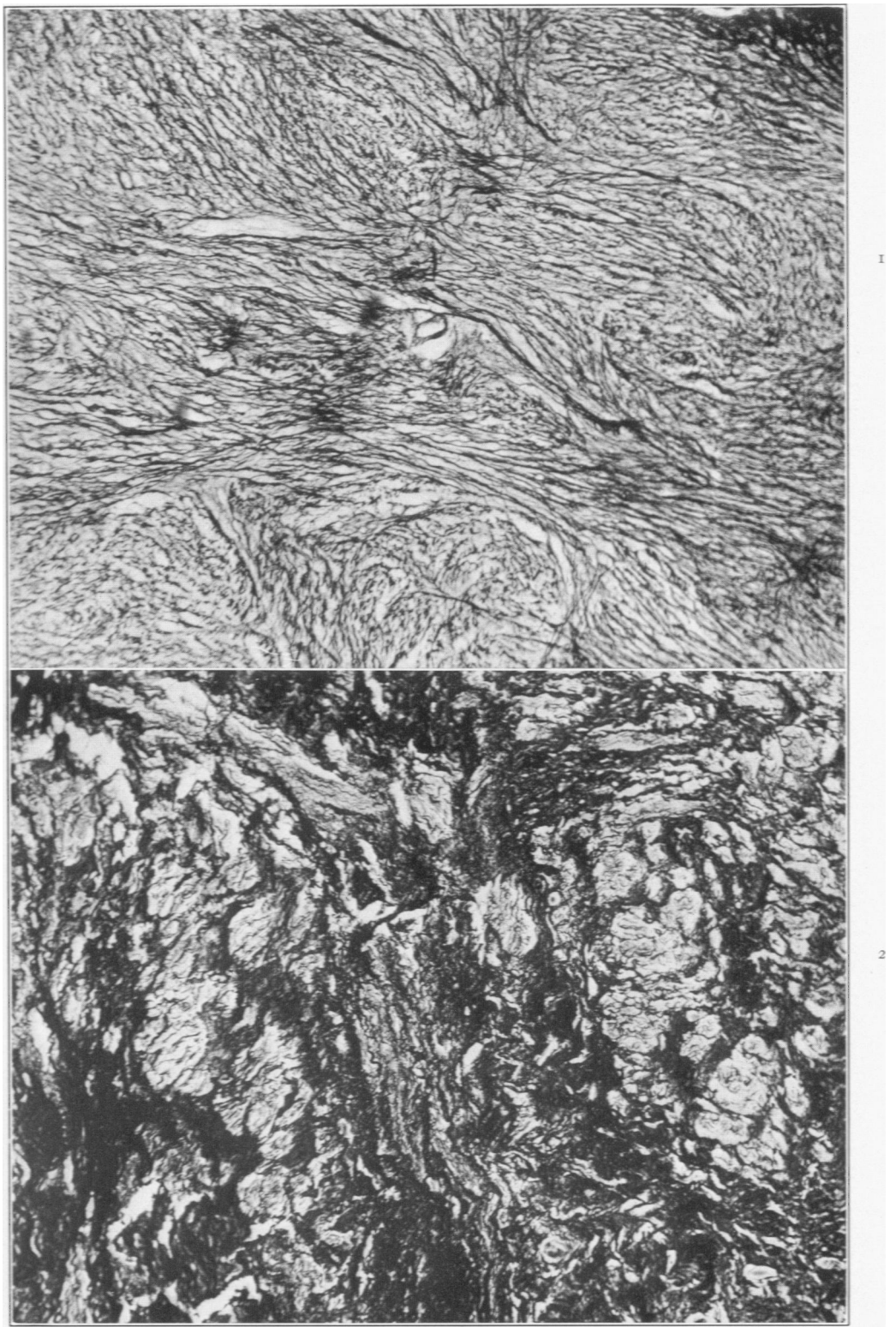
18. Spielmeyer, Walter. Technik der mikroskopischen Untersuchung des Nervensystems. Julius Springer, Berlin, 1924, Ed. 3, 98.

DESCRIPTION OF PLATE

PLATE 101

FIG. 1. Leiomyosarcoma, showing the complex arrangement of the fibrils. × 105.

FIG. 2. Plexiform neurofibrosarcoma. × 170.



Gordon and Sweets Silver Impregnation of Reticulum